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Regulation of Copper Toxicity by *Candida albicans GPA2*

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Copper is an essential nutrient that is toxic to cells when present in excess. The fungal pathogen *Candida albicans* employs several mechanisms to survive in the presence of excess copper, but the molecular pathways that govern these responses are not completely understood. We report that deletion of *GPA2*, which specifies a G-protein α subunit, confers increased resistance to excess copper and propose that the increased resistance is due to a combination of decreased copper uptake and an increase in copper chelation by metallothioneins. This is supported by our observations that a *gpa2*Δ/Δ mutant has reduced expression of the copper uptake genes, *CTR1* and *FRE7*, and a marked decrease in copper accumulation following exposure to high copper levels. Furthermore, deletion of *GPA2* results in an increased expression of the copper metallothionein gene, *CRD2*. Gpa2p functions upstream in the cyclic AMP (cAMP)-protein kinase A (PKA) pathway to govern hyphal morphogenesis. The copper resistance phenotype of the *gpa2*Δ/Δ mutant can be reversed by artificially increasing the intracellular concentration of cAMP. These results provide evidence for a novel role of the PKA pathway in regulation of copper homeostasis. Furthermore, the connection between the PKA pathway and copper homeostasis appears to be conserved in the pathogen *Cryptococcus neoformans* but not in the nonpathogenic *Saccharomyces cerevisiae*.

Copper is an essential nutrient that drives a wide variety of essential biochemical processes through its function as an enzyme cofactor. Excess copper is toxic because it can lead to the generation of reactive oxygen species, via the Fenton reaction, which can, in turn, react with and damage nucleic acids, proteins, and lipids (1, 2). The biocidal properties of copper have been known for centuries and have long been exploited to control or prevent the growth of a wide variety of microbial organisms (3). Furthermore, an emerging theme in the field of host-pathogen interactions is the idea, for which there is considerable evidence, that macrophages kill phagocytized microbes by pumping copper into the phagolysosome to inflict copper poisoning upon them (4–6). The ability of microbes to survive in the presence of excess copper can be achieved by three key mechanisms–reducing copper influx, increasing copper efflux, and expressing metallothioneins, which function as copper storage proteins by chelating excess copper (7–12).

In *Candida albicans*, the major invasive fungal pathogen in humans, copper import is mediated by the high-affinity copper transporter, Ctr1p, and the action of two proteins with cupric reductase activity, Fre7p and Fre10p (13, 14). Under copper-limiting conditions, *CTR1* and *FRE7* are expressed at high levels, and this expression is dependent on the copper-sensing transcription factor encoded by *MAC1*, which binds to copper response elements in the promoters of *CTR1* and *FRE7*. Under copper-replete conditions, the expression of *CTR1* and *FRE7* is repressed (15). Although the mechanism of repression in the presence of copper has not been determined in *C. albicans*, it is likely to occur by the same molecular mechanism as used by *Saccharomyces cerevisiae*, whereby copper binds to and inhibits the ability of ScMac1p to bind DNA and subsequently prevents gene expression (7, 16, 17). (Herein, we use the prefix "Sc" to indicate *S. cerevisiae* gene products, and genes without a prefix are carried by *C. albicans*). As an added barrier to prevent excess copper accumulation, the presence of high copper levels induces the degradation of the copper transporter, ScCtr1p, at the plasma membrane in a manner that is dependent on ScMac1p (11, 18).

Essential for the ability of *C. albicans* to resist copper toxicity is the function of the proteins encoded by *CRP1*, *CUP1*, and *CRD2*. Homozygous deletion of any of these genes results in increased sensitivity to excess copper (19, 20). *CRP1* encodes a P1-type ATPase copper transporter that actively pumps excess copper out of the cell, a feature that appears to be absent in *S. cerevisiae* (19, 20). *CUP1* and *CRD2* encode copper metallothioneins (19, 20). The expression of *CRP1* and *CUP1* is stimulated by growth in excess copper, while *CRD2* expression is insensitive to copper levels (19, 20). The mechanism responsible for the copper-inducible expression of *CUP1* has not been analyzed in *C. albicans*, but it likely mimics the scenario in *S. cerevisiae*, in which copper binds to and activates a second copper-sensing transcription factor, ScCup2, and subsequently induces the expression of *ScCUP1* (21–23). The *C. albicans* genome encodes a strong homologue of Sc*CUP2*, and deletion of either homologue confers copper hypersensitivity to its respective organism (23, 24).

Relatively little is known about the molecular pathways that pathogenic fungi use to sense and respond to excess copper apart from the function of the two copper-sensing transcription factors. Here we demonstrate that deletion of *GPA2*, which encodes a G-protein α subunit involved in filamentous growth, confers increased resistance to normally toxic levels of copper. We show that Gpa2p governs the expression of genes involved in copper uptake and chelation and provide the first evidence for the involvement of the protein kinase A (PKA) pathway in copper homeostasis.

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TABLE 1 Strains used in this study

Strain	Relevant genotype	Reference
C. albicans		
RBY1179	*GPA2/GPA2*	26
RBY1166	*gpa2*::*HIS1*/*gpa2*::*LEU2*	26
RBY1205	*gpa2*::*HIS1*/*gpa2*::*LEU2*/*GPA2*::*SAT1-FLIP* (*addback*)	26
CAI4-URA	*EFG1/EFG1*	43
DSC10	*efg1*Δ::*hisG*/*efg1*Δ::*hisG*	43
DSC11	*efg1*Δ::*hisG*/*efg1*Δ::*hisG*::*EFG1-dpl200*	43
SN250	*MAC1/MAC1 CUP2/CUP2*	24
TF065-1	*mac1*Δ::*HIS1*/*mac1*Δ::*LEU2*	24
TF065-2	*mac1*Δ::*HIS1*/*mac1*Δ::*LEU2*	24
TF039-1	*cup2*Δ::*HIS1*/*cup2*Δ::*LEU2*	24
TF039-2	*cup2*Δ::*HIS1*/*cup2*Δ::*LEU2*	24
S. cerevisiae		
BY4741[a]	*GPA2*	44
*gpa2*Δ	*gpa2*Δ	44
C. neoformans		
M049[b]	*GPA1*	37
AAC1	*gpa1*Δ::*ADE2*	37
AAC2	*gpa1*Δ::*ADE2* + pGPA1 on Cn Tel-Hyg	37

[a] The genotype of *S. cerevisiae* strain BY4741 is MAT**a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*.
[b] The genotype of the *C. neoformans* strains M049 is *MAT*α *ade2*.

TABLE 2 qRT-PCR oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)
CTR1-qF	TCTTTGTTGCCTTCCTTGCT
CTR1-qR	GTTCCACGAGCTTTTGTGGT
CTR2-qF	TGGATCACAATATGCCTGGA
CTR2-qR	ACCCAATGTTGCCAATTCAT
CRP1-qF	GCGCACAGCTGATTTGATTA
CRP1-qR	CCACAAGGACATGCAACAAC
CRD2-qF	CTGCTCAATGTGTCTGTGCTC
CRD2-qR	CACAAATAGCATTAGCACCACAA
CUP2-qF	ACAACTGACCCTGCCAAATC
CUP2-qR	TACCACTACCAGCACCACCA
CUP9-qF	ATGCTCCATTGCTTCCTCAT
CUP9-qR	TGGTTGTTGTGGTTGTTGCT
MAC1-qF	ACCGACCCTGAAACAACAAG
MAC1-qR	AGACGAATCTGCTGGAGGAA
FRE7-qF	TCCACGGTAAGTGATGGTCA
FRE7-qR	ACCGGCAATAAGACCACAAG
CUP1-qF	TTAACTACGCATCTGGCTGC
CUP1-qR	TTGCATTCAGTTTCGGAAGC

MATERIALS AND METHODS

Strains and media. *Candida albicans*, *Saccharomyces cerevisiae*, and *Cryptococcus neoformans* strains were routinely passaged in YPD (2% dextrose, 2% Bacto peptone, 1% yeast extract) at 30°C. All strains are listed in Table 1.

Growth assays. Single colonies were inoculated into 3 ml of YPD and grown overnight in an orbital shaker at 30°C. For liquid growth assays, the overnight cultures were diluted in YPD and used to inoculate 200-μl cultures of YPD (with or without the addition of $CuSO_4$ [Sigma], cisplatin [Sigma], and/or 10 mM N^6,2′-*O*-dibutyryl-AMP [dbcAMP] [Sigma]) at a starting optical density at 600 nm (OD_{600}) of 0.01 in a microtiter plate. The cultures were grown at 30°C with orbital shaking in a microplate reader (Infinite M200 Pro [Tecan]) which recorded OD_{600} readings at specific times. For *C. albicans* and *S. cerevisiae* spot assays on solid media, overnight cultures were diluted in YPD to an OD_{600} of 5 and were then serially diluted 1:5 in YPD. Three microliters of each serial dilution was then spotted onto the appropriate plates, incubated at 30°C, and photographed. Spot assays using the *C. neoformans* strains were performed as described above except the overnight cultures were diluted to an OD_{600} of 0.05 and were then used as a starting point for 2-fold serial dilutions.

Gene expression analysis. Single colonies were inoculated into 3 ml of YPD and grown overnight at 30°C. These overnight cultures were used to inoculate 100-ml cultures of YPD, which were incubated at 30°C with orbital shaking until mid-log phase (OD_{600}, ~1), at which point the cultures were split into two 50-ml aliquots. $CuSO_4$ was added to one of the aliquots to a final concentration of 12 mM. The other aliquot was left untreated. Each culture was allowed to grow for an additional 30 min before being harvested by centrifugation. Pellets were stored at −80°C until RNA extraction. Total RNA was extracted from each frozen pellet using the Ambion RiboPure yeast kit (Invitrogen) and was treated with the Turbo DNA-free kit (Invitrogen) followed by reverse transcription (RT) using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturers' instructions. The cDNA was subject to quantitative reverse transcription-PCR (qRT-PCR) using Power SYBR green PCR master mix (Applied Biosystems). Reactions were run and measurements were obtained using the 7900 Fast Real-Time PCR System and SDS2.3 software (Applied Biosystems). Quantitative RT-PCR primers for 8 genes (*CTR1*, *CTR2*, *CRP1*, *CUP1*, *CRD2*, *MAC1*, *CUP2*, and *FRE7*) were designed using the Primer3 software. The sequence of each oligonucleotide is listed in Table 2.

ICP-MS. Single colonies were inoculated into 3 ml of YPD and grown overnight at 30°C. These overnight cultures were used to inoculate 100-ml cultures of YPD, which were incubated at 30°C with orbital shaking to mid-log phase (OD_{600}, ~1) at which point the cultures were split into two 40-ml aliquots. $CuSO_4$ was added to one of the aliquots to a final concentration of 12 mM. The other aliquot was left untreated. Each culture was allowed to grow at 30°C for 1 h and then harvested by centrifugation. Pellets were washed 3 times with 40 ml of deionized water before being stored at −80°C until inductively coupled plasma mass spectrometry (ICP-MS) analysis. Frozen pellets were resuspended in 20% trace metal-grade nitric acid (Fisher Scientific) at room temperature and then boiled overnight (~16 h) at 100°C in screw-cap Eppendorf tubes. The samples were then transferred to 50-ml conical tubes, and the Eppendorf tubes were rinsed with 2% trace metal-grade nitric acid in triplicate to ensure complete transfer of metal ions. An internal standard was incorporated into each sample to verify calibration of the instrument. Samples were brought to a final volume of 25 ml by addition of 2% trace metal-grade nitric acid. All ICP-MS analyses were carried out on an Agilent 7700 instrument, using the semiquantitative mode. All samples were measured in triplicate, corrected for differences in the number of cells in each sample as measured by OD_{600}.

RESULTS AND DISCUSSION

Deletion of *C. albicans GPA2* confers resistance to copper. During our studies to define the functions of novel unannotated transcripts in the *C. albicans* genome, we observed that a strain harboring a deletion of the transcript listed as NOVEL-Ca21chr3-018 (25) displayed increased growth relative to a wild-type (WT) strain on rich media supplemented with excess copper sulfate ($CuSO_4$) (data not shown). Since this transcript is in close proximity (350 bp) to the start codon of *GPA2* on chromosome 3 and is transcribed on the same strand, we reasoned that deletion of this transcript might confer increased copper tolerance by reducing the expression of *GPA2*. To directly address this, we tested the ability of a strain carrying a homozygous deletion of *GPA2* (26) to grow in the presence of excess copper. The *gpa2*Δ/Δ strain exhibited significant growth in liquid medium supplemented with $CuSO_4$, while the growth of the WT strain was significantly inhib-

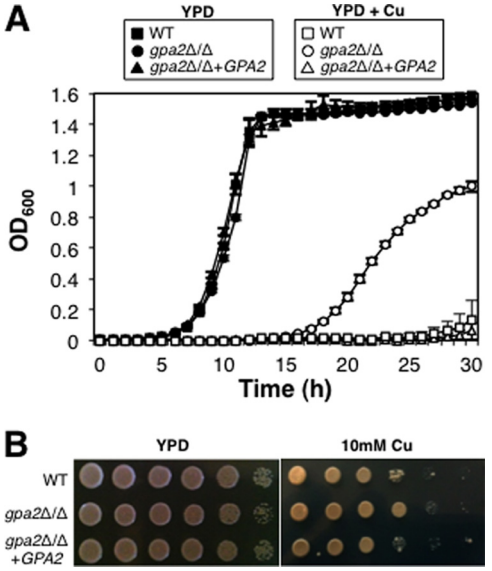

FIG 1 A *gpa2*Δ/Δ mutant is resistant to copper. (A) *C. albicans* WT (squares; RBY1179), *gpa2*Δ/Δ (circles; RBY1166), and *gpa2*Δ/Δ *GPA2* (triangles; RBY1205) strains were inoculated into YPD (closed symbols) or YPD plus 12 mM $CuSO_4$ (open symbols) at an OD_{600} of 0.01. The ODs were measured hourly over the course of 30 h. Shown are the averages of 3 biological replicates. (B) *C. albicans* WT (RBY1179), *gpa2*Δ/Δ (RBY1166), and *gpa2*Δ/Δ *GPA2* (RBY1205) strains were grown overnight in YPD, serially diluted, and spotted onto YPD or YPD plus 10 mM $CuSO_4$ and photographed after 1 or 2 days, respectively.

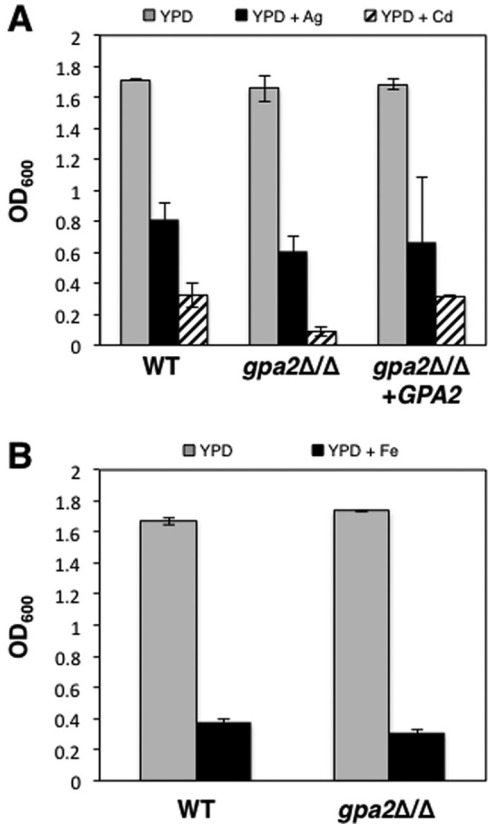

FIG 2 A *gpa2*Δ/Δ mutant is not resistant to excess silver, cadmium, or iron. (A) *C. albicans* WT (RBY1179), *gpa2*Δ/Δ (RBY1166), and *gpa2*Δ/Δ *GPA2* (RBY1205) strains were inoculated into YPD (gray bars), YPD plus 75 μM Ag_2SO_4 (black bars), or YPD plus 100 μM $CdSO_4$ (striped bars) at an OD_{600} of 0.01. ODs were measured at 24 h. Shown are the averages of 3 biological replicates, with standard deviations. (B) *C. albicans* WT (RBY1179) and *gpa2*Δ/Δ (RBY1166) strains were inoculated into YPD or YPD plus 12 mM $FeSO_4$ at an OD_{600} of 0.01. ODs were measured at 24 h. Shown are the averages of 3 biological replicates.

ited (Fig. 1A). Like that of the WT strain, the growth of the complemented strain (26), generated by introducing a WT copy of *GPA2* into the *gpa2*Δ/Δ mutant, was inhibited in the presence of copper (Fig. 1A), indicating that deletion of *GPA2*, and not an extraneous mutation, is responsible for the observed copper tolerance. Similar results were obtained from growth tests performed on solid media in which we observed a 5-fold increase in copper-resistant growth in the *gpa2*Δ/Δ mutant that was reversed in the complemented strain (Fig. 1B).

We observed that the copper resistance of the *gpa2*Δ/Δ strain was more severe than that of the strain harboring the deletion of NOVEL-Ca21chr3-018 (data not shown), suggesting that deletion of NOVEL-Ca21chr3-018 partially reduced the expression of *GPA2*, resulting in a less severe phenotype. Taken together, these results indicate that *GPA2*, and not NOVEL-Ca21chr3-018, functions to govern copper homeostasis in *C. albicans*.

We considered whether or not the copper resistance phenotype of the *gpa2*Δ/Δ was indicative of a general defect in metal homeostasis by testing growth of the mutant in media depleted of or supplemented with other metals. We observed no difference between the WT and the *gpa2*Δ/Δ mutant in growth assays under iron-limiting conditions (500 μM ferrozine or 100 μM bathophenanthrolinedisulfonic acid) or copper-limiting conditions (100 μM bathocuproine disulfonate) or in media containing excess cadmium, silver, iron (Fig. 2), manganese, nickel, or cobalt (data not shown).

A *gpa2*Δ/Δ mutant displays altered regulation of copper-related genes. To determine whether *GPA2* may influence copper resistance through altered regulation of one or more copper-related genes, we assayed the steady-state and copper-inducible expression of several copper-related genes by qRT-PCR in the WT, *gpa2*Δ/Δ mutant, and complemented strains.

The expression of the copper uptake genes, *CTR1* and *FRE7*, was repressed after exposure to excess copper in both the WT and complemented strains (Fig. 3A and B). When only the untreated samples are considered, the *gpa2*Δ/Δ mutant had severely reduced expression of *CTR1* and *FRE7* (11.5-fold and 8.2-fold, respectively) relative to the WT strain, and this reduction was not apparent in the complemented strain. As expected, among the samples that were exposed to excess copper, both genes were expressed at comparably low levels in all 3 strains tested. Therefore, in the absence of excess copper, deletion of *GPA2* confers a decrease in the expression of the copper uptake genes, *CTR1* and *FRE7*.

The expression of the copper-efflux pump, *CRP1*, was induced upon exposure to excess copper in both the WT and complemented strains (Fig. 3C). When only the copper-treated samples are considered, the *gpa2*Δ/Δ strain displayed a 5.2-fold reduction in *CRP1* expression relative to the WT strain that was not evident in the complemented strain (Fig. 3C). *CRP1* was expressed at equivalently low levels in each of the 3 strains when they were left untreated (Fig. 3C). Therefore, deletion of *GPA2* results in the

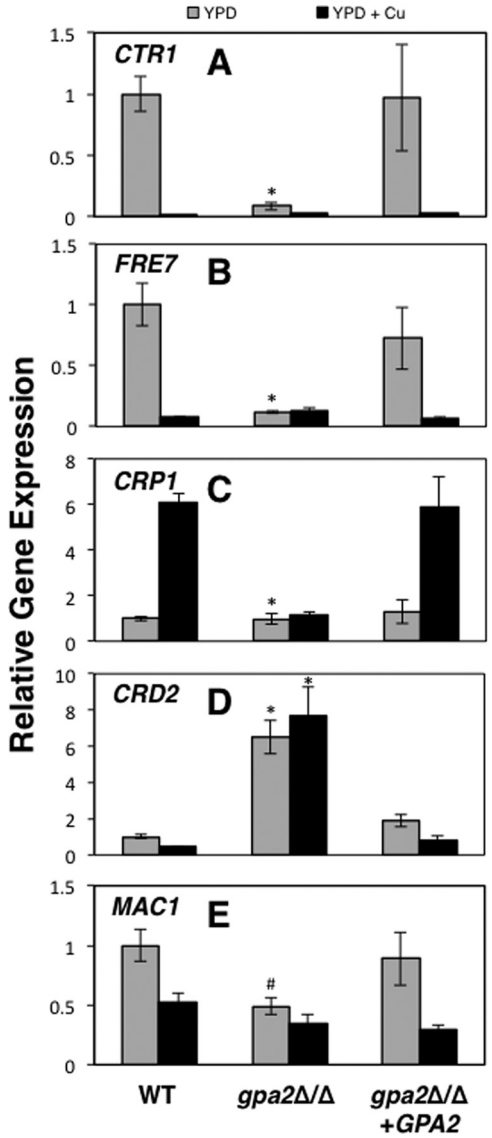

FIG 3 Altered expression of copper-related genes in a *gpa2*Δ/Δ mutant. WT (RBY1179), *gpa2*Δ/Δ (RBY1166), and *gpa2*Δ/Δ *GPA2* (RBY1205) strains grown in YPD (gray bars) or YPD plus 12 mM CuSO4 for 30 min (black bars) were subjected to qRT-PCR expression analysis of the indicated copper-related genes: *CTR1* (A), *FRE7* (B), *CRP1* (C), *CRD2* (D), and *MAC1* (E). RNA expression was normalized to *TEF1* expression, and fold changes between strains were normalized to the WT strain (RBY1179) grown in the absence of copper, which was adjusted to a value of 1. Shown are the averages of 4 biological replicates, with standard errors. *, $P < 0.01$ compared to the WT strain grown under the same condition. #, $P < 0.05$ compared to the WT strain grown under the same condition.

inability of the *CRP1* gene to be induced by exposure to excess copper.

The expression of the metallothionein gene, *CRD2*, did not respond to the addition of excess copper in any of the strains tested (Fig. 3D). Notably, under both conditions, the *gpa2*Δ/Δ strain exhibited increased expression (6.5-fold and 17.8-fold) relative to the WT strain, and this increased expression was not evident in the complemented strain. Therefore, deletion of *GPA2* results in increased in expression of the copper metallothionein gene, *CDR2*.

The expression of the gene encoding the copper-sensing transcription factor, *MAC1*, was repressed after exposure to excess copper in both the WT and complemented strains (Fig. 3E). Among the copper treated samples, *MAC1* was expressed at comparable levels in all three strains (Fig. 3E). However, *MAC1* expression was reduced 2-fold in the untreated *gpa2*Δ/Δ sample relative to the untreated WT sample, and this reduction was not evident in the complemented strain (Fig. 3E). Therefore, in the absence of excess copper, deletion of *GPA2* results in a decrease in *MAC1* expression.

We were unable to detect a reproducible, statistically significant difference in the expression of *CUP1*, *CTR2*, or *CUP2* between the WT and *gpa2*Δ/Δ strains (data not shown). Taken together, the results of our gene expression analyses suggest that the increased copper resistance observed in the *gpa2*Δ/Δ mutant results from a combination of decreased copper uptake and increased synthesis of metallothioneins. Although our results suggest that increased copper efflux is not a factor contributing to the copper resistance phenotype, the possibility exists that a previously undescribed posttranscriptional mechanism could be increasing the amount of efflux pumps without altering the amount of RNA message.

The decreased expression of *CTR1* (Fig. 3A), *FRE7* (Fig. 3B), and *MAC1* (Fig. 3E) in the *gpa2*Δ/Δ mutant is likely to reflect a decrease in expression of the copper-responsive transcription factor, Mac1p, which activates transcription in the absence of excess copper by binding to copper responsive elements (CuREs) in the promoters of its target genes (15). We examined the expression of *CTR1* in independently generated strains that each harbor a homozygous deletion of *MAC1* (24). As expected, the *mac1*Δ/Δ strains exhibited significantly reduced expression of *CTR1* (Fig. 4A) compared to the wild-type strain.

A *gpa2*Δ/Δ mutant accumulates less copper. To extend upon the changes we observed in the expression of the copper uptake genes, *CTR1* and *FRE7*, we tested the strains' sensitivity to cisplatin, a chemotherapeutic drug that is toxic to yeast cells. In *S. cerevisiae* and mammals, cisplatin enters cells through the copper transporter encoded by *ScCTR1* (30). A *S. cerevisiae* deletion of *ScCTR1* displays increased resistance to cisplatin (30). Although the mechanism by which cisplatin is taken up into *C. albicans* cells has not been elucidated, we reasoned that it is likely to be mediated by the *C. albicans CTR1* gene, given its sequence and functional homology to the Sc*CTR1* gene of *S. cerevisiae* (31). Thus, an increased resistance to cisplatin would imply a decrease in expression or function of the *C. albicans CTR1* gene. Consistent with the gene expression data, the *gpa2*Δ/Δ strain displayed increased growth in the presence of cisplatin compared to WT and complemented strains (Fig. 5). Therefore, deletion of *GPA2* results in an increase in resistance to cisplatin. This result is consistent with the notion that a decrease in copper uptake is a contributing factor in the copper resistance of the *gpa2*Δ/Δ strain.

The cisplatin (Fig. 5) and copper (Fig. 1) resistance phenotypes, as well as the decrease in *CTR1* gene expression (Fig. 3A) in the *gpa2*Δ/Δ strain, led us to examine the cell-associated copper content of these strains. To accomplish this, we performed inductively coupled plasma mass spectrometry (ICP-MS) on the WT, *gpa2*Δ/Δ, and complemented strains grown in YPD alone or supplemented with 12 mM $CuSO_4$. All three of the strains had equivalent, low levels of cell-associated copper when grown in YPD. As expected, treatment of WT cells with $CuSO_4$ for 1 h resulted in a significant increase (175-fold) in the cellular copper content

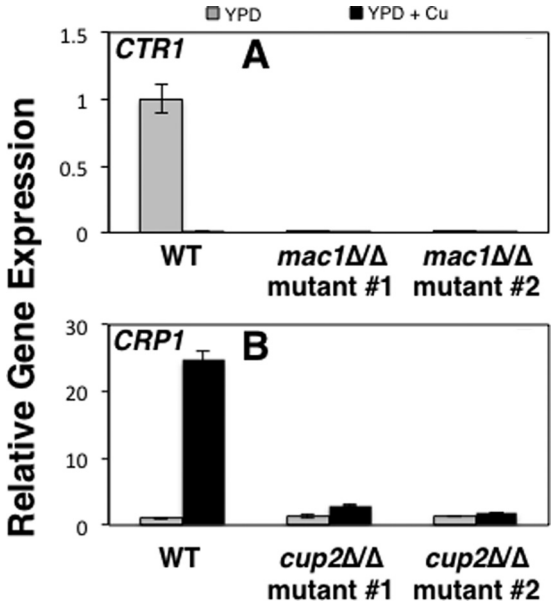

FIG 4 Altered expression of copper-related genes in a *mac1*Δ/Δ mutant and a *cup2*Δ/Δ mutant. (A) The WT (SN250) and 2 independently generated *mac1*Δ/Δ mutants (24) were grown in YPD (gray bars) or YPD plus 12 mM CuSO4 for 30 min (black bars) and were subjected to qRT-PCR expression analysis of *CTR1*. (B) The WT (SN250) and 2 independently generated *cup2*Δ/Δ mutants (24) were grown in YPD (gray bars) or YPD plus 12 mM CuSO4 for 30 min (black bars) and were subjected to qRT-PCR expression analysis of *CRP1*. RNA expression was normalized to *TEF1* expression, and fold changes between strains were normalized to the WT strain (SN250) grown in the absence of copper, which was adjusted to a value of 1. Shown are the averages of at least 3 biological replicates, with standard errors.

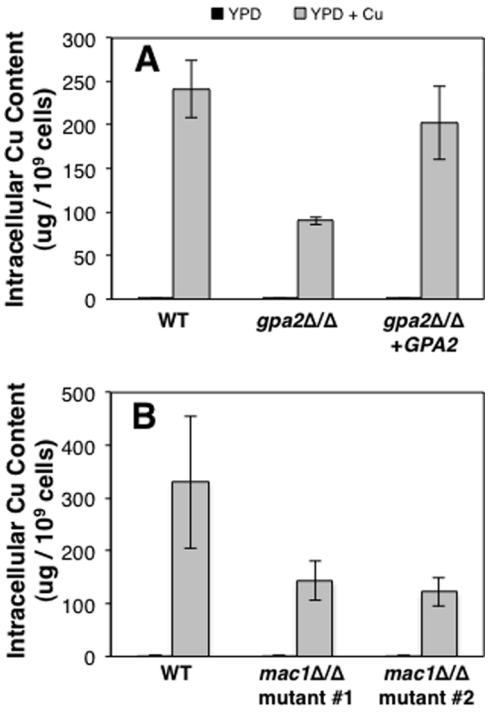

FIG 6 A *gpa2* null mutant accumulates lower levels of copper. (A) *C. albicans* WT (RBY1179), *gpa2*Δ/Δ (RBY1166), and *gpa2*Δ/Δ *GPA2* (RBY1205) strains were grown to mid-log phase in YPD. (B) The *C. albicans* WT (SN250) and 2 independently generated *mac1*Δ/Δ mutants were grown to mid-log phase in YPD. The cultures were divided and grown in YPD (black bars) or YPD plus 12 mM $CuSO_4$ (gray bars) for 1 h. The cells were washed 3 times with water and then boiled in nitric acid overnight prior to ICP-MS. The copper concentrations were normalized to the OD_{600} of each culture. Shown are the averages of 3 biological replicates.

(Fig. 6A). However, following exposure to 12 mM $CuSO_4$ for an hour, the *gpa2*Δ/Δ mutant had reduced (2.1-fold) copper content compared to those of the WT and complemented strains (Fig. 6A). Therefore, deletion of *GPA2* results in a decrease in cell-associated copper content upon exposure to copper. This result is consistent with the increased cisplatin resistance and the decreased *CTR1* expression that we have observed for the *gpa2*Δ/Δ mutant. Together, these observations favor the model that reduced copper uptake contributes to the copper resistance phenotype of the *gpa2*Δ/Δ mutant.

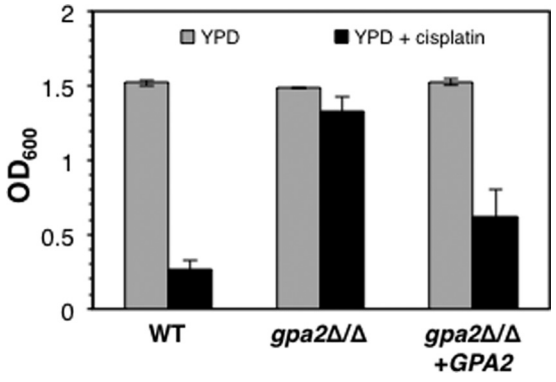

FIG 5 A *gpa2* null mutant is resistant to cisplatin. *C. albicans* WT (RBY1179), *gpa2*Δ/Δ (RBY1166), and *gpa2*Δ/Δ *GPA2* (RBY1205) strains were inoculated into YPD (gray bars) or YPD plus 600 μM cisplatin (black bars) at an OD_{600} of 0.01. The ODs were measured at 24 h. Shown are the averages of 3 biological replicates.

We noticed a difference in *CTR1* and *FRE7* expression between the WT and *gpa2*Δ/Δ strains under conditions where no extra copper was added to the medium but not under conditions of excess copper (Fig. 3A and B), where we observed the difference in copper accumulation (Fig. 6). This difference can be explained by copper-inducible protein degradation. *S. cerevisiae* Ctr1p is degraded at the plasma membrane when cells are exposed to high levels of copper (11, 18). While this aspect of regulating Ctr1p function in *C. albicans* has not been authenticated, it is reasonable to predict a similar role. Based on the gene expression data, one likely scenario is that the *gpa2*Δ/Δ strain has severely reduced steady-state levels of Ctr1p. Thus, the amount of Ctr1p that needs to be degraded to adequately shut down copper import is much lower, allowing the *gpa2*Δ/Δ strain to turn off copper import more rapidly and subsequently achieve lower copper levels than the WT strain. Further experiments are required to conclusively determine this mechanism.

The decreased accumulation of cell-associated copper in the *gpa2*Δ/Δ mutant is most likely the result of decreased expression of *MAC1* in the *gpa2*Δ/Δ mutant, which, as demonstrated above, confers decreased expression of the copper transporter, *CTR1* (Fig. 4A). We measured the amount of cell-associated copper in the *mac1*Δ/Δ strains grown in YPD alone or YPD supplemented with 12 mM $CuSO_4$. Deletion of *MAC1* confers a defect in cell-

associated copper accumulation (~2-fold decrease) very similar to that conferred by deletion of *GPA2* (Fig. 6). These results are consistent with the model in which deletion of *GPA2* leads to reduced copper accumulation by decreasing the expression of the copper-sensing transcription factor *MAC1*.

The expression of *CRP1* is induced when excess copper is added to the medium (19, 20). The decreased accumulation of copper in the *gpa2*Δ/Δ strain (Fig. 6A) might explain the lack of induction of the copper efflux pump, *CRP1* (Fig. 3C). In *S. cerevisiae*, copper-inducible gene expression is mediated by the function of *ScCUP2*. Under conditions where copper is in excess, *Sc*Cup2p binds to copper and activates the expression of *ScCUP1*, which specifies a metallothionein (21–23, 32, 33). The *C. albicans CUP2* homologue is required to resist high copper levels because deletion of this gene confers hypersensitivity to excess copper (24), making it a likely candidate for the transcription factor that mediates the copper-inducible expression of *CPR1*. To test this in *C. albicans*, we examined the copper-inducible gene expression of *CRP1* in two independently generated strains that each harbor a homozygous deletion of the *CUP2* gene (24). As expected, the *cup2*Δ/Δ strains exhibited significantly reduced copper-induced expression of *CRP1* (Fig. 4B) compared to the wild-type strain. To our knowledge, this is the first report that connects *CRP1* expression to the activity of *CUP2* in *C. albicans*.

Copper resistance can be reversed by cyclic AMP. The *C. albicans GPA2* gene encodes a G-protein α subunit that functions with *GPR1*, a G-protein coupled receptor, as a nutrient sensor that regulates filamentous growth (34–36). *GPA2* in hyphal morphogenesis is known to act through the cAMP-dependent PKA pathway, as deletion of *GPA2* results in a morphogenesis defect that can be reversed by the addition of exogenous cAMP to the medium (34). To determine if *GPA2* functions through the same pathway to govern copper homeostasis, we tested whether the addition of 10 mM N^6,2′-*O*-dibutyryl-AMP (dbcAMP) would reverse the copper resistance of the *gpa2*Δ/Δ strain. dbcAMP is a cell-permeative, nonmetabolic derivative of cAMP that has previously been used to mimic high cAMP levels in *C. albicans* (35). Addition of dbcAMP did not affect the growth of the WT or *gpa2*Δ/Δ strain in the absence of copper (Fig. 7). While the *gpa2*Δ/Δ mutant achieved significant growth in YPD supplemented with $CuSO_4$ (Fig. 1 and 7A), its growth was significantly inhibited in same medium to which dbcAMP was added (Fig. 7A). Therefore, addition of exogenous cAMP can bypass the effect on copper tolerance of the *GPA2* deletion and render the *gpa2*Δ/Δ strain sensitive to copper. *EFG1* encodes a transcription factor that functions at the end of the cAMP-PKA pathway to govern hyphal morphogenesis. In order to further investigate the role of the PKA pathway in copper toxicity, we tested whether mutations in the *EFG1* branch of the *C. albicans* PKA pathway would confer increased resistance to excess copper. We observed that an *efg1*Δ/Δ mutant was significantly more resistant to excess copper than both the WT and the *EFG1* complemented strain (Fig. 7B). This result is in agreement with the findings of Homann et al. (24). Taken together, these results suggest that *GPA2* functions through the PKA pathway to govern copper homeostasis.

Conservation of *GPA2* function among pathogenic yeasts. We next considered whether this new role for *GPA2* in copper homeostasis is conserved in other fungi. We decided to address this by analyzing deletion mutants in the nonpathogenic *S. cerevisiae* and the primary pathogen *Cryptococcus neoformans*. The *GPA2* homologues in *S. cerevisiae* and *C. neoformans* are Sc*GPA2* and Cn*GPA1*, respectively. Both genes have been shown to function upstream of the cAMP-PKA pathway in their respective organisms (37–41). We could not detect a difference between the WT *S. cerevisiae* strain and the *gpa2*Δ mutant in the presence of excess copper when grown in liquid or on solid medium (Fig. 8A and 8B, respectively). Therefore, deletion of Sc*GPA2* does not appear to confer copper resistance, suggesting that *GPA2* does not govern copper homeostasis in *S. cerevisiae*. However, we cannot exclude the possibility that another gene provides redundant function in the Sc*gpa2*Δ mutant.

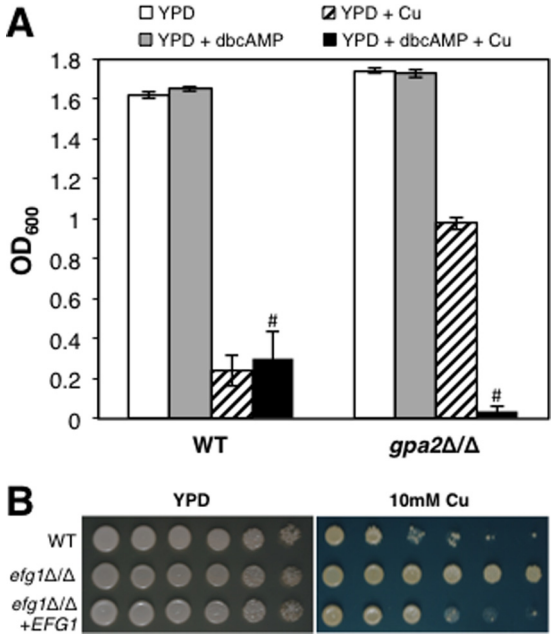

FIG 7 Role of the cAMP-PKA pathway in copper resistance. (A) *C. albicans* WT (RBY1179) and *gpa2*Δ/Δ (RBY1166) strains were inoculated into YPD (white bars), YPD plus dbcAMP (gray bars), YPD plus 12 mM $CuSO_4$ (striped bars), or YPD plus dbcAMP plus 12 mM CuSO4 (black bars) at an OD_{600} of 0.01. ODs were measured at 24 h. Shown are the averages of 3 biological replicates. (B) *C. albicans* WT (CAI4-URA), *efg1*Δ/Δ (DSC10), and *efg1*Δ/Δ *EFG1* (DSC11) strains were grown overnight in YPD, serially diluted, and spotted onto YPD or YPD plus 10 mM $CuSO_4$ and photographed after 1 or 2 days, respectively. #, no statistically significant difference between these two samples.

The *C. neoformans gpa1*Δ strain exhibited an ~5-fold increase in growth in the presence of excess copper relative to the WT strain, and the resistance was reversed in the complemented strain carrying a WT copy of *GPA1* (Fig. 8C). Therefore, deletion of *GPA1* confers copper resistance to a similar extent as deletion of *C. albicans GPA2*.

There are several observations in the literature that are consistent with deletion of Cn*GPA1* resulting in decreased copper import. Alspaugh et al. demonstrated that Cn*GPA1* regulates capsule production and melanization by showing that a strain harboring a deletion in Cn*GPA1* displays defects in these processes (37). In a study performed 14 years later, Silva et al. observed that treatment of *C. neoformans* cells with microplusin, a copper-chelating antimicrobial peptide, inhibited both capsule production and melanization (42). One possible

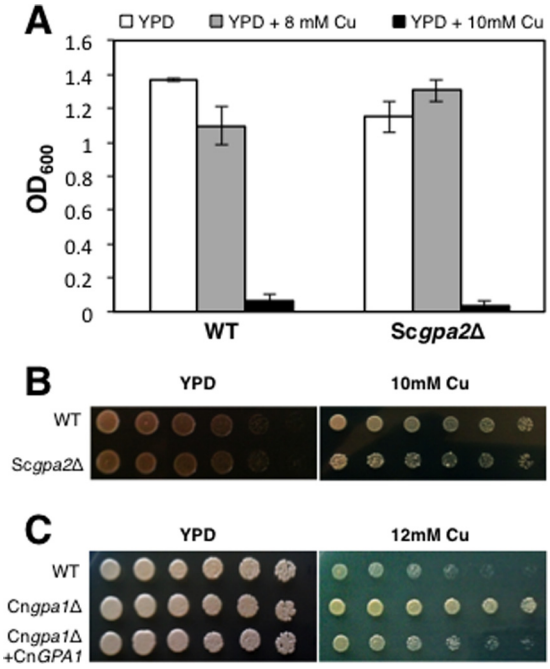

FIG 8 Relationship between *GPA2* and copper toxicity in other fungi. (A) *S. cerevisiae* WT (BY4741), and *gpa2*Δ strains were inoculated into YPD (white bars), YPD plus 8 mM $CuSO_4$ (gray bars), or YPD plus 10 mM $CuSO_4$ at an OD_{600} of 0.01. The ODs were measured at 40 h. Shown are the averages of 3 biological replicates. (B) *S. cerevisiae* strains WT (BY4741) and *gpa2*Δ strains were grown overnight in YPD, serially diluted, and spotted onto YPD or YPD plus 10 mM $CuSO_4$ for 1 or 2 days, respectively. (C) *C. neoformans* WT (M029), *gpa1*Δ (AAC1), and *gpa1*Δ *GPA1* (AAC3) strains were grown overnight in YPD, serially diluted, and spotted onto YPD or YPD plus 12 mM $CuSO_4$ and photographed after 1 or 2 days, respectively.

explanation for the similarities in phenotypes shared by deletion of Cn*GPA1* and depletion of copper by chelation is that a Cn*gpa1*Δ mutant has a defect in copper import, similar to what we observed in *C. albicans*. More experiments are required to reach this conclusion definitively.

Relationship between *GPA2* and copper homeostasis. When exposed to toxic concentrations of copper in the environment, *C. albicans* employs three key microbial mechanisms to ensure survival. It responds by reducing copper influx, increasing copper efflux, and expressing metallothioneins that function to chelate free copper ions (19, 20). Our understanding of the signaling pathways that mediate these responses is incomplete, with most of the knowledge being extrapolated from observations made in *S. cerevisiae*. In this report, we describe the novel role of *GPA2* and the PKA pathway in copper homeostasis. We demonstrate that a strain harboring a deletion in *GPA2* results in a copper resistance phenotype (Fig. 1) that stems from 2 of the 3 key copper survival mechanisms–decreased copper import (Fig. 3, 5, and 6) and increased methallothionein expression (Fig. 3D). The *GPA2* gene encodes a G protein α subunit that functions through the cAMP-PKA pathway to govern hyphal morphogenesis (34, 35). Our observation that the copper resistance can be reversed by addition of dbcAMP suggests that *GPA2* also functions through the cAMP-PKA pathway to govern copper homeostasis, presumably by controlling the expression of the copper import genes, *CTR1* and *FRE7*, as well as the metallothionein gene, *CRD2*. Further experiments are required to determine the exact pathways and mechanisms that *GPA2* uses to control the expression of these genes.

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